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# Gut Microbial Metabolism and Nonalcoholic Fatty Liver Disease

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The gut microbiome, the multispecies community of microbes that exists in the gastrointestinal tract, encodes several orders of magnitude more functional genes than the human genome. It also plays a pivotal role in human health, in part due to metabolism of environmental, dietary, and host-derived substrates, which produce bioactive metabolites. Perturbations to the composition and associated metabolic output of the gut microbiome have been associated with a number of chronic liver diseases, including nonalcoholic fatty liver disease (NAFLD). Here, we review the rapidly evolving suite of next-generation techniques used for studying gut microbiome composition, functional gene content, and bioactive products and discuss relationships with the pathogenesis of NAFLD. (*Hepatology Communications* 2019;3:29-43).

The human gastrointestinal tract harbors trillions of bacterial, fungal, and archaeal cells in addition to viral particles. Collectively termed the “gut microbiome,” members of these microbial communities engage each other and the human host by exchanging signaling molecules and substrates, interactions that are now emerging as critically important in defining host health.<sup>(1)</sup> (Table 1) The healthy gut microbiome comprises a large diversity of phylogenetically distinct microbial species, with even greater interindividual diversity observed at the subspecies or strain level.<sup>(2)</sup> Although culture-independent studies of the gut microbiome have more traditionally focused on bacteria, investigation of fungal and viral species has increased, leading to their recognition as integral members of the gut microbiome

and implicating trans-kingdom interactions in microbiome composition and function.<sup>(3,4)</sup>

The healthy adult gut microbiome encodes several orders of magnitude more functional genes than the human genome, considerably expanding the functional capacity of the human superorganism.<sup>(5)</sup> Whereas average genomic differences between individual humans are small, the unique collection of microbial species and strains within an individual's gut microbiome confers a high degree of interpersonal functional capacity.<sup>(6)</sup> As the field of gut microbiome research has developed, it has become clear that the gut microbiome plays a pivotal role in defining host health status. This is achieved through more traditional host–microbial interactions involving expression of virulence factors and production of

*Abbreviations:* AAA, aromatic amino acid; BCAA, branched-chain amino acid; FMT, fecal microbial transplantation; FXR, farnesoid X receptor; LC, liquid chromatography; mRNA, messenger RNA; MS, mass spectrometry; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

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**TABLE 1. DEFINITIONS OF KEY TERMS**

Term	Definition
Microbiota	Microbes (e.g., bacteria, fungi, viruses) that collectively inhabit an ecosystem.
Microbiome	Collection of all genomes of microbes in an ecosystem.
Biomarker-based sequencing	Process of cataloguing microbes through analysis of sequence variation in a single ubiquitous gene.
Whole metagenome sequencing	Process of cataloguing all the genetic material present in a biologic sample.
Metatranscriptomics	Process of profiling all expressed genes (RNA) in a biologic sample.
Metaproteomics	Study of all proteins found in a biologic sample.
Metabolomics	Study of all small-molecule chemicals found in a biologic sample.

cell-associated ligands (e.g., lipopolysaccharides) that influence microbial sensing and response systems (e.g., toll-like receptors), which function as immune gatekeepers.<sup>(7)</sup> However, it is increasingly recognized that the products of microbial primary and secondary metabolism also modulate host cellular physiology and immune responses, thus offering an additional avenue by which the gut microbiome and its products may influence host health. Among the known gut microbiome products that influence host cell responses are short-chain fatty acids (SCFAs) that serve as energy sources for host cells, interspecies communication or quorum-sensing molecules, complex macrolide and non-ribosomal peptides with antimicrobial and immunomodulatory capacity, as well as vitamins and neuro-signaling molecules.<sup>(1,8)</sup>

That the gut microbiome is central to human health is underscored by an ever-increasing body of literature indicating that perturbations to the composition, function, or metabolic output of the gut microbiome are associated with a wide array of chronic disease states.<sup>(1)</sup>

Given that the liver receives the majority of its blood supply from the intestine through the portal circulation, it is particularly exposed to gut microbiome-derived products. Unsurprisingly, the gut microbiome has been implicated in the development of chronic liver diseases, including nonalcoholic fatty liver disease (NAFLD).<sup>(9)</sup> Here, we review the following:

- Current methods for studying gut microbiome composition, function, and metabolic productivity.
- Factors that shape the gut microbiome.
- Gut microbial metabolism and its effect on human health.
- The influence of gut microbial metabolism in the pathogenesis of NAFLD.
- Potential therapeutic implications of the gut microbiome in the management of NAFLD.

## Current Methods in Microbiome Research

A variety of methods exist for characterizing the gut microbiome. Each of these tools, including advantages and current limitations, is reviewed in Table 2. Sequencing methods include biomarker gene-based sequencing that permit an assessment of microbial community composition as well as untargeted “shotgun” approaches that enable determination of the genes encoded (metagenomics) or expressed (metatranscriptomics) by the microbiome. We also review mass spectrometry (MS)-based platforms that permit identification of proteins (metaproteomics) or metabolites (metabolomics) produced by the microbiome. Each of these methods has been comprehensively reviewed elsewhere,<sup>(10-13)</sup>

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TABLE 2. ADVANTAGES AND LIMITATIONS OF METHODS FOR STUDYING THE MICROBIOME

Method	Advantages	Limitations
Sequencing methods		
Biomarker-based sequencing	<ul style="list-style-type: none"><li>• Useful for samples from sites of low microbial biomass (e.g., mucosal biopsies).</li><li>• Large reference databases exist and enable predictions of functional traits of a community.</li><li>• Least expensive sequencing method.</li></ul>	<ul style="list-style-type: none"><li>• Lack of species-level resolution in taxonomic identification.</li><li>• Functional predictions can be limited.</li><li>• Not applicable to viral communities.</li><li>• May introduce bias secondary to amplification of a single gene.</li><li>• Sensitive to technical failures (e.g., primer choice).</li></ul>
Whole ("shotgun") metagenome sequencing	<ul style="list-style-type: none"><li>• Measures microbial functional genes.</li><li>• Permits parallel microbial taxonomic identification at species and/or strain level.</li></ul>	<ul style="list-style-type: none"><li>• Deep sequencing depths are often required.</li><li>• More expensive and laborious than biomarker-based sequencing.</li></ul>
Metatranscriptomics	<ul style="list-style-type: none"><li>• Evaluates microbial activity and captures dynamic variation in transcriptional activity.</li></ul>	<ul style="list-style-type: none"><li>• Requires careful sample collection and storage.</li><li>• rRNA and host mRNA must be removed.</li><li>• May be biased by both mRNA stability and transcription rates of microbes.</li><li>• Most expensive and laborious sequencing method.</li></ul>
Mass spectrometry-based methods		
Metaproteomics	<ul style="list-style-type: none"><li>• May provide a more accurate view of microbial productivity when compared to metatranscriptomics.</li></ul>	<ul style="list-style-type: none"><li>• Removal of host-derived proteins is not feasible.</li><li>• Protein identification is dependent on reference databases.</li></ul>
Metabolomics	<ul style="list-style-type: none"><li>• Permits identification of the end products of microbial activity.</li><li>• Most likely method to identify disease biomarkers and facilitate discovery of underlying mechanisms of disease.</li><li>• Can be performed as targeted or untargeted analyses.</li></ul>	<ul style="list-style-type: none"><li>• Identification of metabolites are database- and platform-dependent.</li><li>• Does not discriminate between identification of microbial-derived metabolites and those derived from the human host or diet.</li></ul>

and therefore this review provides an overview of each approach (Fig. 1).

BIOMARKER-BASED MICROBIOTA PROFILING

Many studies have catalogued the identity of distinct microbes present in a specimen through determination of sequence variants in a specific polymerase

chain reaction–amplified microbial biomarker gene. Commonly used biomarker genes include the 16S ribosomal RNA (16S rRNA) gene and the internal transcribed spacer 2 region of bacteria and fungi, respectively. Due to the lack of a conserved biomarker gene across known viral species, no comparable approach exists for viruses; their detection currently relies on species-specific genes or the reassembly of viral genomes from shotgun metagenomic sequencing

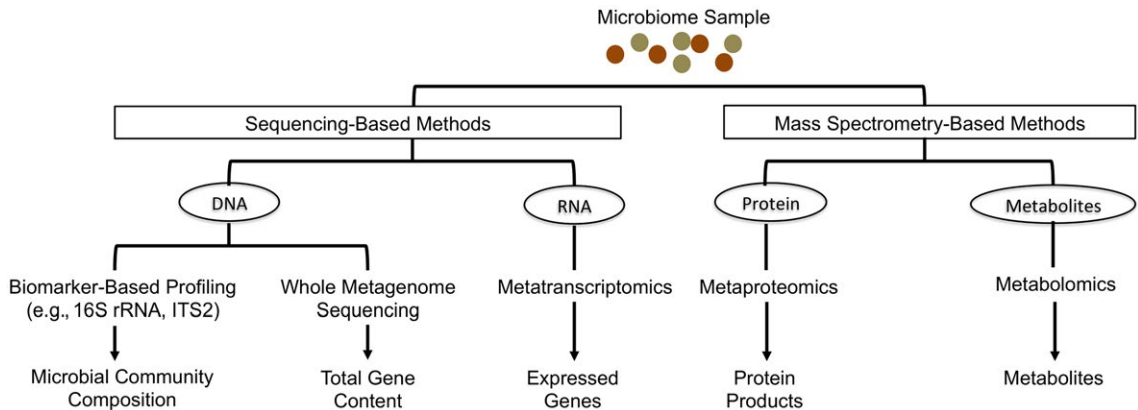


FIG. 1. Methods for studying gut microbiome composition, function, and metabolic productivity.

efforts. Biomarker-based sequencing offers the opportunity to economically characterize microbial community membership, structure, and taxonomic relative abundance and is particularly useful for large sample sizes and when the microbe:host ratio is low (e.g., mucosal biopsies). Inferential algorithms based on reference bacterial genomes enable predictions of evolutionarily conserved functional traits from 16S rRNA sequences.<sup>(14)</sup> Although a useful tool, poor species-level resolution in 16S rRNA gene sequencing, limited availability of closed bacterial genomes, and horizontal gene transfer across microorganisms may limit such *in silico* metagenomic predictions.<sup>(15)</sup>

## WHOLE METAGENOME SEQUENCING

In contrast with biomarker-based sequencing, shotgun metagenomic sequencing does not focus on a single biomarker gene but rather DNA sequences arising from genomes within a microbial community. Sequenced fragments are either mapped to a reference genomic database or undergo *de novo* assembly to produce contiguous regions of microbial genomes, which subsequently undergo functional gene annotation using specialized platforms.<sup>(16)</sup> Thus, metagenomic sequencing permits parallel identification of microbial species and their encoded functional genes within a microbiome. More recent human metagenomic studies have demonstrated the capacity to follow gut microbiomes at strain-level resolution, permitting assessment of microbial population dynamics in the neonatal gut<sup>(17)</sup> and tracking of microbial species (and their functional genes) that engraft following fecal microbial transplantation (FMT).<sup>(18)</sup> Although the cost of sequencing has declined dramatically over the past decade, the need for very large numbers of sequence reads per sample to permit sufficient community coverage and metagenomic reconstruction coupled with the computational needs to facilitate analysis of such large data sets have resulted in shotgun metagenomics being more commonly applied to smaller representative sample sets.

## METATRANSCRIPTOMICS

Although DNA-based metagenomic sequencing permits identification of microbes and encoded genes

within a microbiome, it does not provide information on microbial gene expression. Indeed, even though microbiome perturbation is commonly associated with established disease, progression and severity may be mechanistically linked to changes in the transcriptional program of an otherwise compositionally stable pathogenic microbiome. Metatranscriptomic shotgun sequencing (also commonly referred to as RNA sequencing [RNA-seq]) determines the gene expression profile of a microbiome, which, like other microbiota or microbiome profiling approaches, offers greatest utility when examined longitudinally and when related to specific host conditions (e.g., disease remission or flare) or, for example, in response to dietary inputs.<sup>(19)</sup> Due to the need for high-quality and high-quantity RNA for metatranscriptomics, samples must be appropriately preserved at the time of sample collection, i.e., treated with a nucleic acid preservative. Following RNA isolation, rRNA, which can represent upwards of 75% of total RNA, is selectively depleted to enrich for messenger RNA (mRNA), long noncoding RNA, and microRNA. The mRNA pool is then fragmented and reverse transcribed to produce complementary DNA for sequencing. Bioinformatic tools similar to those used for shotgun metagenomics are then used to map, annotate, and quantify gene expression profiles derived from these data sets.<sup>(16,20)</sup>

Metatranscriptomic analysis permits identification of the specific organisms responsible for expression, even if the specific gene or pathway of interest is more broadly encoded within the metagenome. In addition, it also permits directionality to be determined, i.e., whether host or microbial genes are expressed. The fundamental difference between turnover rates of RNA and DNA (minutes or less versus hours or more) underpins how metatranscriptome profiles may better capture contemporaneous microbiome responses to host exposures. This is particularly true for the gut microbiome, which is frequently exposed to nutritional and xenobiotic substrates, many of which have been shown to exert substantial effects on gut microbiome composition and functional gene capacity.<sup>(21,22)</sup> However, it should be noted that mRNA stability, which is inherently low in prokaryotes,<sup>(23)</sup> may differ across distinct microbial species, leading to differential mRNA degradation across microbes that could skew metatranscriptome profiles.<sup>(24)</sup>



## METAPROTEOMICS

Facilitated by high-throughput MS, metaproteomics profiles the complement of proteins produced by a microbiome, offering a complementary view of microbiome function. Given that proteins are inherently more stable than mRNA and represent the end products of posttranscriptional and posttranslational regulatory mechanisms, this approach may also provide a more accurate view of microbial productivity.

Metaproteomics currently uses both gel-based and gel-independent liquid chromatography (LC) separations prior to tandem MS (MS/MS)-based peptide identification. Whole proteomes obtained following cellular lysis can be fractionated by centrifugation based on cellular localization or chemical properties (e.g., phosphorylation) prior to peptide and protein separation and identification. To identify peptides and proteins, high-throughput proteomics software pipelines<sup>(25)</sup> use search algorithms<sup>(26)</sup> to compare MS/MS spectra to protein databases or to predicted intact masses and fragmentation patterns generated *in silico* from the corresponding metagenome.

Nonuniform peptide ionization and/or selective peptide loss during MS detection makes direct quantification of shotgun proteomic data challenging.<sup>(27)</sup> To address this technical challenge, much effort has been directed toward stable isotope labeling,<sup>(28)</sup> which enables quantification of the abundance of labeled protein under different states, and label-free MS methods,<sup>(29)</sup> which exploit intrinsic MS measurement metrics to quantify proteins. Apart from permitting quantitative proteomics, stable isotope labeling and label-free MS methods have grown in popularity due to their simplicity, relatively low cost, and universal applicability.

## METABOLOMICS

Metabolomics comprises complementary targeted and untargeted analytical chemistry techniques, including nuclear magnetic resonance spectrometry, gas chromatography-MS (GC-MS), and LC-MS, that yield vast amounts of information regarding the composition of both inorganic and organic compounds associated with a microbiome. These include but are not limited to amino acids, lipids, sugars, organic acids, secondary bile acids, and more complex structures, such as macrolides and polyketides. Similar

to metaproteomics, spectra are compared with existing databases to facilitate metabolite identification and, in some cases, source tracking.<sup>(30,31)</sup>

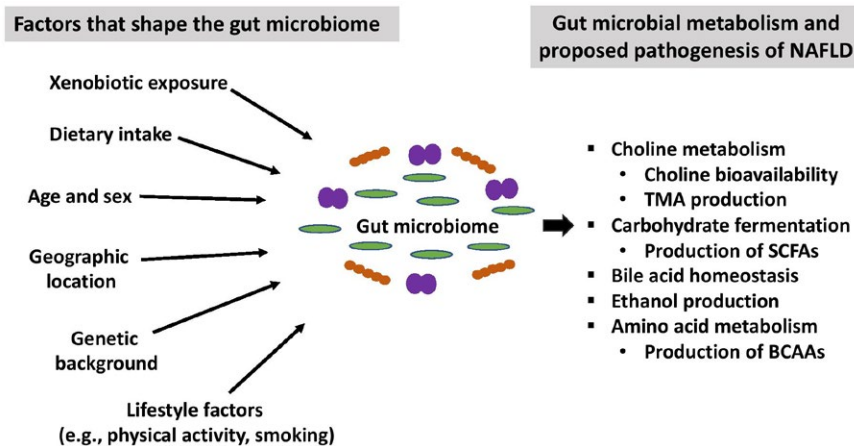
Traditionally, throughput and range of protein and metabolite analyses lag far behind that of nucleic acid sequencing platforms (current platforms are predominantly based on MS analyses coupled to slow front-end separations, such as GC and LC) and are hobbled by relatively underdeveloped databases. Fortunately, recent advances in rapid gas-phase ion mobility spectrometry separations, which distinguish molecular conformations, have helped to decrease the long front-end separation times and increase throughput of metabolite detection.<sup>(32)</sup> In parallel, efforts in the field are focused on expanding current databases.

## INTEGRATING “OMICS” DATA

No single “omics” approach can fully decipher a mechanistic pathway in disease pathogenesis, and combining results from different platforms strengthens insight into microbiome–host interactions.<sup>(33)</sup> The fundamental challenge in “multi-omic” approaches is that these processes generate immense amounts of multidimensional data. As such, novel statistical methods for integrated data analysis that combine disparate data sets and standardized quality control metrics are constantly evolving. In addition to challenges with analytical approaches, data storage associated with such data sets requires robust computational resources and infrastructure. However, as the field advances and data sets increase in complexity and complementarity, the application of systems biology approaches promises to expand our mechanistic understanding of the gut microbiome across temporal and health gradients.

## Factors That Shape the Gut Microbiome

Microbiome studies, especially those performed in human subjects, must account for potential confounding factors; therefore, an understanding of the factors that shape the gut microbiome is critical to experimental design.<sup>(34)</sup> (Fig. 2) The healthy gut microbiome rapidly diversifies over the first several years of life,<sup>(35,36)</sup> typically remains compositionally



**FIG. 2.** Factors that shape the gut microbial community and postulated mechanistic pathways linking gut microbial metabolism to the development of NAFLD.

stable in adulthood,<sup>(37,38)</sup> and exhibits diversity loss in the elderly.<sup>(39)</sup> The stability of functional gene content encoded by the gut microbiome is less well studied. Mehta et al.<sup>(19)</sup> recently performed metagenomic and metatranscriptomic sequencing of stool samples collected 6 months apart from 308 adults to examine temporal stability of gut microbial function. Although strain composition and the core microbial metagenome were relatively stable over time, unsurprisingly, transcriptomic variance exceeded metagenomic variance. These findings suggest that a single DNA-based measurement of the adult gut microbiome can likely yield reliable information regarding composition and gene content but that the metatranscriptome (and thus the associated metabolic products) is more dynamic and related to contemporary exposures.

Although the host genome has been shown to influence the composition of the gut microbiome,<sup>(40,41)</sup> environmental influences play a more significant role in shaping the human gut microbiome.<sup>(42)</sup> In studies of the gut microbiota of monozygotic twins, fewer than half of detected bacterial taxa are shared, indicating that a minority of microbial taxa are inherited.<sup>(35)</sup> Apart from host genetics, a number of endogenous and exogenous factors shape microbial communities in the gut. These include dietary and alcohol intake,<sup>(43,44)</sup> smoking,<sup>(45,46)</sup> sex and hormonal cycles,<sup>(47)</sup> diurnal variation,<sup>(48)</sup> physical activity,<sup>(49)</sup> and xenobiotic exposure (including antibiotics).<sup>(22,50)</sup>

A combined analysis of large patient cohorts from three continents (Europe, Asia, and North America) revealed country-specific microbial signatures, suggesting that culture and geographic location significantly impact the gut microbiome composition and functional genes it encodes.<sup>(51)</sup> These geographic microbial signatures are also known to be related to differences in diet, with functional gene enrichments in both babies and adults across geographically distinct sites closely linked with the proportion of plant polysaccharides and simple sugars consumed in the diet.<sup>(35,52-54)</sup> Indeed, short-term dietary interventions in healthy adults modulate the gut microbiota, in part due to providing a competitive advantage to gut microbes capable of extracting energy from the specific dietary components consumed.<sup>(1)</sup> Although there is currently no standardized approach to account for dietary data in human microbiome studies, this is an area of ongoing investigation.<sup>(55)</sup> Nevertheless, there is sufficient compelling evidence to suggest that dietary intake along with other environmental factors that may shape the gut microbiome must be accounted for in human microbiome studies.

## The Gut Microbiome in NAFLD

NAFLD, the liver manifestation of obesity and the metabolic syndrome, encompasses a spectrum of

histopathologic abnormalities ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH, a more severe phenotype of disease, is associated with increased risk for development of cirrhosis, hepatocellular carcinoma, and liver-related mortality.<sup>(56)</sup> Among the 100 million adults with NAFLD in the United States, 15% to 20% have NASH.<sup>(13)</sup> Despite the overwhelming burden of NAFLD, there are no approved pharmacologic therapies for NAFLD in the United States.<sup>(57)</sup> Although the mechanisms that lead to the development of NAFLD and progression to NASH are not fully elucidated, an ever-growing body of literature suggests that the gut microbiome is implicated in the pathogenesis of NAFLD.

Studies in animal models have provided the most compelling evidence for a causal role of the gut microbiome in the pathogenesis of NAFLD. Germ-free mice lacking a gut microbiome are resistant to the development of hepatic steatosis,<sup>(58)</sup> and FMT can transmit hepatic steatosis in murine models.<sup>(59)</sup> Moreover, administration of probiotics or antibiotics to mice, which can dramatically alter gut microbiome composition and has also been shown to influence metabolic output in humans,<sup>(60)</sup> inhibits development of high-fat diet-induced hepatic steatosis.<sup>(61,62)</sup>

A number of cross-sectional human studies have demonstrated an association between gut microbiota perturbation and the full clinical spectrum of NAFLD, including nonalcoholic fatty liver without steatohepatitis (NAFL),<sup>(63-71)</sup> NASH,<sup>(64,65,68,69,72-76)</sup> NAFLD-related advanced fibrosis (fibrosis stage >2),<sup>(68,76-79)</sup> and NAFLD-related cirrhosis and hepatocellular carcinoma.<sup>(77)</sup> Several of these studies were performed in pediatric cohorts,<sup>(63,64,75)</sup> and an overwhelming majority of these studies used biomarker-based sequencing to profile the gut microbiota. The majority of these studies revealed a decrease in overall bacterial diversity in NAFLD, similar to that reported in obesity.<sup>(80)</sup> However, as for other chronic diseases,<sup>(81-83)</sup> human microbiome studies in NAFLD have not identified a unique microbial signature. The most consistent finding among studies is that higher disease severity, and in particular advanced fibrosis, is associated with enrichment of members of the *Bacteroides* and *Escherichia* genera. Moreover, the gut microbiome in adults with NAFLD-related cirrhosis as compared to adults with cirrhosis secondary to other etiologies comprises a higher abundance of *Porphyromonadaceae* and *Bacteroidaceae* and lower abundance of *Veillonellaceae*.<sup>(84)</sup>

Discordant results between studies could be attributable to a variety of factors, such as differences in patient age (adults versus children/adolescents) and comorbidities (e.g., body mass index, diabetes), geographic location, definition of NAFLD clinical phenotypes, and microbial functional redundancy as well as technical differences in stool sample handling, sequencing, or statistical analysis methods between studies. This is particularly pertinent because many of the published studies did not measure or adjust for factors known to affect the gut microbiome. The inability to find a distinct disease-associated microbiota signature is not unique to NAFLD<sup>(82)</sup> and may also be attributable to a failure to detect overrepresented and underrepresented microbial taxa when statistical analyses are limited to diversity indices and/or comparisons at the phylum or family level (the predominant analytical approaches employed in NAFLD studies). Secondly, microbial biomarker sequencing studies fail to provide information on the functional gene content or products of the microbiome, thus potentially missing the crucial microbial signature associated with disease. This was demonstrated in a study of lean and obese individuals who were better differentiated by their gut metagenome as opposed to their microbiota profile.<sup>(85)</sup> Finally, recent biomarker sequencing studies of large patient cohorts have demonstrated that several distinct microbiota conformations are evident in patients with a given clinical indication and are related to differences in immunologic profiles and clinical features of disease.<sup>(81,86)</sup> This implies that within a given diseased patient population several pathogenic microbiomes may exist; however, such possibilities remain unexplored in the field of NAFLD microbiome research.

Among the NAFLD human microbiome studies performed to date, a few have used one or more omic techniques and thus have added new perspectives on functional attributes of the gut microbiome in NAFLD pathogenesis.<sup>(63,64,69,70,75,78,79)</sup> Da Silva et al.<sup>(69)</sup> performed targeted profiling of eight fecal metabolites of interest and found that a higher concentration of two fecal SCFAs, propionate and isobutyric acid, differentiated adults with NAFL from healthy controls. Del Chierico et al.<sup>(64)</sup> performed an integrated analysis of the gut microbiota (by 16S rRNA-seq) and gut metabolome (by targeted metabolomics) in a pediatric cohort with the



following clinical phenotypes: NAFL, NASH, and obese and healthy controls. In multivariate analysis, children with NASH had lower levels of the genus *Oscillospira*, increased levels of the genera *Dorea* and *Ruminococcus*, and higher levels of two gut metabolites, 2-butanone and 4-methyl-2-pentanone, when compared to controls.

Loomba et al.<sup>(79)</sup> were the first to examine the whole gut metagenome, which was integrated with serum metabolomics, in adults with NAFLD. The prospective cohort study included 86 patients with biopsy-proven NAFLD, of which 72 had no or minimal fibrosis (stages 0-2) and 14 had advanced fibrosis (stages 3-4). Whereas differential abundances in microbial gene pathways and serum metabolites did not achieve statistical significance after correction for multiple testing, 37 bacterial species, including *Escherichia coli* and *Bacteroides vulgatus*, were differentially represented between minimal and advanced fibrosis phenotypes. When these bacterial species were incorporated into a model with patient age, body mass index, and a microbial diversity index, the model possessed an area under the receiver operating characteristic curve of 0.936 for detecting advanced fibrosis. This finding is concordant with a prior observation that *B. vulgatus* is a contributor to insulin resistance.<sup>(87)</sup> A more recent study by Caussy et al.<sup>(78)</sup> identified a shared gene effect of serum 3-(4-hydroxyphenyl)lactate, a microbial-derived metabolite, with NAFLD-related advanced fibrosis. Although the functional significance of this metabolite is unknown, 3-(4-hydroxyphenyl)lactate was strongly correlated with the abundance of seven bacterial species that were previously associated with advanced fibrosis, including *B. caccae*, *E. coli*, and *Clostridium sporogenes*.<sup>(79)</sup>

## Gut Microbial Metabolism and Interactions With the Human Host

Gut microbial metabolites have now been linked with specific disease states: autism spectrum disorder (4-ethylphenylsulfate)<sup>(88)</sup> and obesity-associated hepatocellular carcinoma (deoxycholic acid)<sup>(89)</sup> in murine models as well as childhood atopic asthma (12,13-dihydroxy-9Z-octadecenoic acid [12,13 Di-HOME])<sup>(90)</sup>

and cardiovascular disease (trimethylamine-*N*-oxide [TMAO])<sup>(91)</sup> in human subjects. A review of the current evidence of the causal link between the gut microbiota and cardiovascular disease provides an example of how mechanistic approaches to studying the gut microbiome not only yield insight into disease pathogenesis but may also provide the foundation by which microbiome-targeted therapies can be developed.

Gut microbial metabolism of dietary methylamines contributes to the formation of the following three metabolites: choline, betaine, and trimethylamine (TMA).<sup>(92)</sup> TMA is metabolized by the liver to generate TMAO, and manipulation of the gut microbiota, either through FMT or administration of specific bacterial strains, alters TMAO levels in murine models.<sup>(93,94)</sup> In recent years, many human studies have examined the association between serum levels of the microbial-derived metabolite TMAO and cardiovascular disease. Elevated serum TMAO is associated with the degree of atherosclerotic burden and risk of incident cardiovascular events among adults presenting with chest pain and those undergoing coronary angiography.<sup>(95,96)</sup> More recent evidence suggests that elevated serum TMAO levels are also linked to adipose tissue dysfunction<sup>(97)</sup> and chronic kidney disease.<sup>(98)</sup> Subsequent mechanistic studies identified the biologic function of TMAO to include endothelial dysfunction and enhancement of platelet hyperreactivity.<sup>(99,100)</sup> Oral application of a structural analogue of choline, 3,3-dimethyl-1-butanol, inhibited microbial TMA production, lowered serum TMAO levels, and prevented development of atherosclerotic lesions, despite a proatherosclerosis diet, in an animal model.<sup>(101)</sup> Together, these data support that a microbial-derived metabolite is an important contributor to a variety of interrelated cardiometabolic diseases and that targeting the gut microbial capacity to produce this metabolite offers a potential novel strategy for disease prevention or treatment.

## Gut Microbial Metabolism in the Pathogenesis of NAFLD

Unlike in cardiovascular disease, precise microbiome-host mechanisms in NAFLD are not well

delineated. However, based on animal studies, many of which were performed in models of obesity and insulin resistance, several potential mechanistic links between the gut microbiome and NAFLD have emerged. Postulated mechanisms include dysregulation of methylamine metabolism, carbohydrate fermentation and generation of SCFAs, bile acid metabolism, endogenous ethanol production, and amino acid metabolism. The gut microbiota may also contribute to NAFLD through intestinal barrier dysfunction and lipopolysaccharide-induced activation of toll-like receptor pathways of innate immune signaling,<sup>(102)</sup> but for the purpose of this review, discussion is limited to disease mechanisms potentially mediated by microbial metabolism (Fig. 2).

## METHYLAMINE METABOLISM

In addition to the role in cardiovascular disease, gut microbial metabolism of dietary methylamines, specifically choline, may also contribute to the development of NAFLD. Choline deficiency contributes to the development of fatty liver disease through multiple mechanisms, including abnormal phospholipid synthesis, defective very low-density lipoprotein secretion, and alterations in the enterohepatic circulation of bile acids.<sup>(103)</sup> Susceptibility to choline deficiency was previously attributed to factors such as low dietary choline intake, estrogen status, and single-nucleotide polymorphisms in specific host genes for choline metabolism.<sup>(103)</sup> However, the discovery of gut microbial metabolism of choline to produce TMA provides an additional mechanism for low choline bioavailability.

A high-fat diet leads to increased gut microbial metabolism of choline and the development of hepatic steatosis in mice.<sup>(104)</sup> Manipulation of dietary choline intake in 15 female human subjects resulted in variations in the abundance of choline-metabolizing gut microbial species; abundance of these specific microbes along with a single-nucleotide polymorphism of a host choline metabolism gene accurately predicted the degree to which subjects developed fatty liver while on a choline-deficient diet.<sup>(105)</sup> Subsequent human studies have revealed varying associations between by-products of methylamine metabolism and NAFLD severity,<sup>(106,107)</sup> but additional studies are necessary to decipher the exact role of gut microbial methylamine metabolism in NAFLD pathogenesis.

## FERMENTATION OF INDIGESTIBLE CARBOHYDRATES AND PRODUCTION OF SCFAS

Complex carbohydrates undergo microbial fermentation predominantly in the colon, resulting in the production of SCFAs, including acetate, propionate, and butyrate. SCFAs provide the majority of energy needs for intestinal epithelial cells, but they also cross the intestinal epithelial barrier and mediate a variety of biologic activities, including modulation of immune function and exerting antiproliferative effects, in part by signaling through G protein-coupled receptors in multiple tissue sites.<sup>(108,109)</sup>

Fecal SCFA concentrations are increased in genetically obese mice,<sup>(80)</sup> and germ-free mice with low levels of gut SCFAs are protected from diet-induced obesity.<sup>(110)</sup> These data suggest that significantly elevated fecal SCFAs, indicative of increased energy extraction from dietary input, are a hallmark of obesity. However, in contrast to these findings, an increase in butyrate-producing bacteria has been shown to prevent diet-induced hepatic steatosis and bile acid dysregulation and its associated hepatitis in murine models.<sup>(111,112)</sup> Moreover, in humans, increasing colonic propionate prevented weight gain in overweight adults,<sup>(113)</sup> and the beneficial metabolic effects of FMT from lean to obese donors were primarily associated with an increased abundance of butyrate-producing bacterial strains in the gut.<sup>(114)</sup> These contradictory findings are likely attributable to differing end-organ effects of individual or combinations of SCFAs, including varying effects on energy expenditure, production of satiety hormones, and central appetite regulation.<sup>(108)</sup>

## BILE ACID TRANSFORMATIONS BY THE GUT MICROBIOTA

Bile acids are steroid-derived molecules synthesized in the liver and secreted into the intestinal lumen that facilitate lipid absorption and additionally regulate metabolism by activation of a number of host receptors, including farnesoid X receptor (FXR) and the membrane-associated G protein-coupled receptor (TGR5).<sup>(115)</sup> Distinct bile acids have varying affinities for host receptors and may act either as receptor agonists or antagonists, with subsequent effects on the regulation of basal metabolism and energy

expenditure, lipid metabolism, nitric oxide synthesis, intestinal motility and permeability, and immune response.<sup>(116,117)</sup>

In addition to their effects on the host, bile acids prevent intestinal bacterial overgrowth, both directly through membrane-damaging effects and indirectly through induction of antimicrobial protein expression; thus, they also play an important role in shaping gut microbiome membership.<sup>(118)</sup> Indeed, microbial bile acid resistance offers a fitness advantage in the gut and is commonly complemented by the capacity to deconjugate bile acids and convert primary bile acids to secondary bile acids. Germ-free mice or antibiotic-treated mice exhibit low concentrations of conjugated bile acids, pointing to a central role of gut microbiome in regulating bile acid composition.

Animal models have revealed that lean and obese mice have a differential abundance of various bile acids.<sup>(119)</sup> Human studies have noted elevated total serum bile acids, with an altered ratio of secondary to primary bile acids, in adults with NAFLD.<sup>(73,120,121)</sup> Of interest, administration of the FXR agonist obeticholic acid led to improvement in NASH and insulin sensitivity in randomized controlled trials in adults, indicating an important role for bile acid signaling in NAFLD and the potential for therapeutic intervention targeting bile acid receptors.<sup>(122,123)</sup> Thus, dysregulation of the reciprocal relationship between the human gut microbiome and bile acids has the capacity to significantly modulate FXR and TGR5 signaling, modulating host metabolism and immunity, and offers an attractive mechanistic hypothesis linking the gut microbiome and metabolic diseases, such as NAFLD.<sup>(124)</sup> For a comprehensive overview of gut microbiome–bile acid crosstalk in the pathogenesis of metabolic disease, we direct the reader to two recent reviews.<sup>(115,125)</sup>

## ENDOGENOUS ETHANOL PRODUCTION

Hepatic genes involved in alcohol metabolism, including alcohol dehydrogenase, are up-regulated in NAFLD.<sup>(126,127)</sup> A number of studies have demonstrated that children and adolescents with NAFLD have increased serum ethanol levels,<sup>(63,64,75)</sup> although this finding is not consistent across all studies.<sup>(69)</sup> The gut microbiome can generate ethanol from dietary precursors, and manipulation

of the gut microbiome is known to alter endogenous ethanol production.<sup>(128)</sup> A common finding in NAFL and NASH is enrichment in *Escherichia* and/or *Lactobacillus* genera,<sup>(65,68–71,74,75,77–79)</sup> which have the capability to produce ethanol. These findings suggest that increased endogenous ethanol production by the gut microbiome could contribute to the pathogenesis of NAFLD. However, a recent study demonstrated that elevated serum ethanol may also be attributed to insulin-dependent impairments of alcohol dehydrogenase activity in the liver, independent of endogenous alcohol production by the gut microbiome,<sup>(129)</sup> pointing to the existence of distinct disease mechanisms that may converge on a common clinical manifestation and are plausibly linked to gut microbiome functional differentials within this patient population.

## AMINO ACID BIOSYNTHESIS AND METABOLISM

The gut microbiome influences amino acid homeostasis, in part due to biosynthesis and metabolism of aromatic amino acids (AAAs) and branched-chain amino acids (BCAAs). Elevated serum BCAA, postulated to be secondary to perturbations in gut microbial metabolism, has been identified as a potential biomarker for insulin resistance in several cohort studies.<sup>(130,131)</sup> Specifically, two bacterial species, *Prevotella copri* and *B. vulgatus*, are associated with enriched biosynthetic potential for BCAAs and a reduced potential for BCAA transport into bacterial cells in adults with insulin resistance.<sup>(87)</sup> Intriguingly, a more recent study found that *B. vulgatus* was one of the most abundant gut microbes in adults with NAFLD-related advanced fibrosis.<sup>(79)</sup>

Perturbations in microbial metabolism of AAAs and BCAAs and ensuing alterations in the serum metabolite profile have more recently been identified in NAFLD.<sup>(70,132)</sup> Hoyles et al.<sup>(70)</sup> performed an integrated analysis of the gut metagenome, hepatic transcriptome, and serum and urine metabolomes in a cohort of women with obesity but without diabetes. Compared to those without NAFL, women with NAFL had significant alterations in the gut metagenome, including differences in BCAA and AAA pathways, as well as in the serum metabolome. Specifically, serum phenylacetic acid,

an AAA-derived microbial metabolite, was strongly correlated with hepatic steatosis in this cohort. This metabolite was subsequently found to induce hepatic steatosis both *in vitro* (primary culture of human hepatocytes) and *in vivo* (murine model), suggesting a potential causal role in NAFL pathogenesis.<sup>(70)</sup> This study provides a proof-of-concept prototype of how integrated multi-omic analyses in human subjects combined with *in vitro* and *in vivo* mechanistic studies can facilitate identification of possible microbial-driven mechanistic pathways in NAFLD.

## Gut Microbiome-Targeted Therapy in the Management of NAFLD

The prospect of targeting the gut microbiome in the management of NAFLD offers a novel strategy for disease prevention or management. A diverse array of strategies to manipulate the gut microbiome exist and include dietary interventions, probiotics (culture of living microorganisms), prebiotics (fermentable dietary fibers that stimulate the growth and survival of probiotics), synbiotics (combination of probiotics and prebiotics), antibiotics, and FMT.<sup>(1)</sup> Moreover, bariatric surgery results in rapid and sustained shifts in the gut microbiome.<sup>(133)</sup> Damms-Machado et al.<sup>(134)</sup> found that the gut microbiome in adults with obesity who underwent bariatric surgery shifted within 6 months after surgery toward a microbial profile found in adults with normal body weight. Recent studies have revealed the existence of distinct and niche-specific microbial communities along the length of the gastrointestinal tract and that the composition of these communities influence successful engraftment of exogenous microbes,<sup>(135,136)</sup> suggesting that future microbial interventions may be personalized based on an individual's gut microbiome. However, large-scale interventional trials with therapies targeting the gut microbiome in NAFLD are lacking, and a better understanding of the complexities of microbial metabolism and microbe–host interactions is necessary to inform the development of gut microbiome-targeted therapies for this patient population.

## Conclusions

An ever-growing body of preclinical and clinical literature supports that changes to the composition, function, and/or metabolic output of the gut microbiome are associated with NAFLD; however, the exact mechanistic links remain elusive. Integrated multi-omic analyses, based on high-throughput sequencing and MS platforms, are now yielding more comprehensive views of gut microbiome functional capacity and biochemical output in NAFLD. Additional longitudinal prospective cohort studies, which are designed to account for various environmental factors that can shape the gut microbiome and are complemented by *in vitro* and *in vivo* mechanistic studies, are necessary to delineate microbiome–host interactions that contribute to NAFLD pathogenesis. Improved knowledge of the contribution of gut microbial metabolism to liver health could yield the foundation for the development of novel microbiota-targeted precision interventions for the treatment or management of NAFLD.

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